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Erythroid Cell-Specific Determinants of α-Globin mRNA Stability

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Although globin mRNAs are considered prototypes of highly stable messages, the mechanisms responsible for their longevity remain largely undefined. As an initial step in identifying potential *cis*-acting elements or structures which contribute to their stability, we analyzed the defect in expression of a naturally occurring α2-globin mutant, $\alpha^{\text{Constant Spring}}$ (CS). The CS mutation is a single-base change in the translation termination codon (UAA→CAA) that allows the ribosome to read through into the 3′ nontranslated region (NTR). The presence of CS mRNA in transcriptionally active erythroid precursors and its absence (relative to normal α-globin mRNA) in the more differentiated transcriptionally silent erythrocytes suggest that this mutation disrupts some feature of the α-globin mRNA required for its stability. Using a transient transfection system, we demonstrate that in murine erythroleukemia cells the CS mRNA is unstable compared with the normal α2-globin mRNA. The analyses of several other naturally occurring and site-directed mutant α-globin genes in murine erythroleukemia cells indicate that entry of a translating ribosome into the 3′ NTR targets the message for accelerated degradation in erythroid cells. In contrast, both the CS and α2-globin mRNAs are stable in several nonerythroid cell lines. These results suggest that translational readthrough disrupts a determinant associated with the α2-globin 3′ NTR which is required for mRNA stability in erythroid cells.

While the major roles of transcription and processing in the control of gene expression have long been acknowledged (13), a number of systems which demonstrate a primary role for regulation at the level of mRNA stability have recently been described (reviewed in references 2, 7, 42, and 44). In eucaryotes, mRNAs have a wide range of half-lives, from as short as a few minutes to as long as several days. These differences in mRNA stability can have significant impacts on the levels of gene expression (20, 48). A variety of elements and mechanisms appear to be responsible for modulating mRNA half-lives. Features governing the turnover of individual mRNAs have been attributed to specific *cis*-acting RNA sequences and structures and to *trans*-acting proteins (10, 27, 36, 41, 45, 46, 55, 57, 63). An additional level of complexity is possible as an mRNA is translated, since the interaction of *cis*- and *trans*-acting elements with the ribosome and its associated components or with higher-order RNA structures could modulate mRNA turnover (47, 52).

The α- and β-globin gene clusters are useful model systems to study mechanisms responsible for mRNA stability. Globin genes are expressed in highly specialized, terminally differentiated erythrocytes (RBC) and can account for as much as 95% of the soluble proteins (17, 43). During RBC differentiation the nucleus is extruded (17) and globin mRNAs increase from less than 1% to more than 98% of the total mRNA (6). This high-level accumulation of globin mRNA is dependent not only on increased transcription but also on the long half-lives of globin mRNAs, 24 to 60 h, and the degradation of nonglobin mRNAs (3, 5, 30, 34, 49, 50). Because of the cessation of transcription during RBC differentiation and because both alleles of a globin gene are expressed, mutations that decrease mRNA stability result in imbalances in the ratios of the mutant and normal mRNAs in heterozygotes. Differences in these mRNA concentrations often cause imbalances in protein synthesis, and by this criterion, a number of globin mutations which are likely to affect message stability can be identified (26, 51). These mutations can be studied to define the features and mechanisms regulating mRNA stability.

Although some progress has been made in identifying potential *cis*-acting RNA elements, *trans*-acting proteins, and nucleases involved in mRNA degradation in the RBC (4, 30, 31, 35, 49, 61), the bases for the longevities of globin mRNAs and for the degradation of nonglobin mRNAs during RBC differentiation are presently undefined. We have initiated studies to explore the mechanisms which regulate α-globin mRNA stability by examining the defect in expression of a naturally occurring α2-globin mutation, $\alpha 2^{\text{Constant Spring}}$ (CS). The CS mutation is a single-base substitution which changes the translation termination codon into a glutamine (UAA→CAA) and allows the ribosome to translate an additional 31 codons into the 3′ nontranslated region (NTR) (12, 40). Translation termination occurs when the ribosome reaches the next in-frame stop codon (UAA), located within the polyadenylation signal AAUAAA. In vivo the CS mutation results in a complete loss of expression from the affected allele (12). Early studies demonstrated that the defect in expression was not at the translational or posttranslational level (25). In subsequent studies, the presence of CS mRNAs in transcriptionally active erythroid precursors and its absence, relative to normal α2-globin mRNAs, in the more-differentiated transcriptionally silent reticulocytes suggested that this mutation disrupted some feature of the α2-globin mRNA required for its stability (24, 33).

In this paper an experimental approach which can be utilized to express and quantitatively compare the levels of two mRNAs that differ by a single base is described. Our studies demonstrate that the CS mRNA is unstable in erythroid cells and is stable in nonerythroid cells. The mechanism responsible for the erythroid cell-specific destabilization of CS mRNAs requires entry of the translating ribosome into the 3′ NTR. These results are consistent with a model wherein a particular

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structure or determinant is associated with the normal α2-globin 3′ NTR and is disrupted by translational readthrough. To account for the accelerated degradation of CS mRNAs in murine erythroleukemia (MEL) cells and differentiating RBC and the equal stabilities of the CS and α2 mRNAs in the nonerythroid cells, we propose that the presence of an intact 3′ NTR determinant may be functionally important only to cells in the erythroid lineage, where it serves to protect the normal α2-globin mRNA from degradation. This type of mechanism could contribute to the high-level accumulation of globin mRNAs during RBC differentiation by enabling the cell to distinguish between globin and nonglobin mRNAs through the recognition and protection of mRNAs containing an intact 3′ NTR determinant.

MATERIALS AND METHODS

Construction of α-globin expression vectors. The pSV2A neoα2 expression vector containing the human α2-globin gene in a 1,493-bp *Pst*I-*Pst*I DNA fragment has been described previously (59). M13-based site-directed mutagenesis (64) was used to generate the following mutations in the α2-globin gene by using the indicated oligonucleotides (5′→3′): CS, TCCA GCTTGACGGTATT; ACG, GAGAGAACCCACCACGG TC; α2 $^{\text{Koya Doya}}$ (KD), TCCAGCTGAACGGTATT; α2$^{\text{Icaria}}$ (Icaria), TCCAGCTTTACGGTATT; α2 $^{\text{Wayne}}$ (Wayne), CG GTATTTGAGGTCAGC; CS/−2 (a stop codon located two codons 5′ to the CS mutation), TCCAGCTTGACCTTATT TGC; U1, GAGGCTCCTTATTAACGGT; CS1 (CS mutation with translation continuing one codon), GAGGCTCCTT ATTGACGGT; U4 and CS4, AACGGCTACTTAGGCT CCAGC; and U14 and CS14, GAGGGCCCGTACTTAG GCCCAGCG. Mutations were introduced into the expression vector by exchanging mutated segments for normal segments as follows: for ACG, a 1,221-bp *Kpn*I-*Bst*EII fragment, and for all other mutations, a 159-bp *Bst*EII-*Apa*I fragment. Clones containing the mutations were identified by using the ^{32}P-labeled mutagenesis primers as probes (60) and were sequenced to verify the presence of the mutation (53). The chimeric c-*fos* promoter/α2-globin gene construct (pfos/α2) was made by excising the α2-globin promoter and 5′ NTR from pSV2Aneoα2 as a 575-bp *Eco*RI-*Nco*I DNA fragment and replacing it with a 595-bp *Eco*RI-*Nar*I fragment containing the c-*fos* promoter (pβSFosMβg [1]). Two complementary oligonucleotides (sequences available on request) were included in the ligation to reintroduce the α2-globin 5′ NTR. In this construct, the c-*fos* transcription start site coincides with the α2-globin start site. The c-*fos*/CS chimeric gene was made by exchanging a 152-bp DNA fragment containing the CS point mutation for the corresponding region in the pfos/α2-globin expression vector. Plasmids were screened and sequenced as described above.

Electroporation and cell culture. Three cell lines were used for these experiments: a MEL cell line (National Institute of General Medical Sciences, Human Genetic Mutant Cell Repository, GM0086E) and two nonerythroid cell lines, murine fibroblasts (C127) and monkey kidney cells (COS-1). Electroporations were performed exactly as described previously (59) except that semiconfluent C127 and COS-1 cells were trypsinized, washed several times with minimal essential medium (MEM), resuspended at 10^6 cells per ml in MEM, and electroporated at 200 V and room temperature. To make stable cell lines for polysome analysis, the pSV2AneoCS and pSV2Aneoα2 plasmids were linearized at the *Kpn*I site 500 bp 5′ to the α2-globin promoter, and for the RNA half-life measurements, 10 μg each of the pfos/α2 and pfos/CS plasmids were linearized at the *Eco*RI site 5′ to the c-*fos* promoter. Cells were electroporated with the linearized DNAs and 2 days later were placed in selective medium containing 400 μg of G418 per ml. The medium was changed every other day, and at the end of 2 weeks, pools of transformed cells were obtained.

For the time course experiments (see Fig. 3 and 4), the transfected C127 cells were split and plated into separate dishes. For each time point tested, one plate of cells was harvested for RNA, and reverse transcription (RT)-PCR analyses were performed with 20% of the cytoplasmic RNA. The transfected MEL cells (see Fig. 2A) were analyzed in the same way, except that aliquots of these suspension cells were removed from a single flask. As the transfected MEL and C127 cells undergo several cell divisions during the 6-day time course, increasing amounts of cytoplasmic mRNAs were used for the RT-PCR assay. Actinomycin D (10 μg/ml) and cycloheximide (50 μg/ml) were added to cells at 24 h postelectroporation, and an equal volume of ethanol or water, respectively, was added to mock-treated cells. After 3 h of drug treatment, cells were harvested for cytoplasmic RNAs. Transfected MEL cells were induced to differentiate by incubating the cells at 2 h postelectroporation in medium containing 1.8% dimethyl sulfoxide (16) supplemented with bovine serum albumin (38, 58). Fresh induction medium was added daily. Stable cell lines containing the *fos*/CS or *fos*/α2-globin gene constructs were made quiescent by a 3-day incubation in MEM containing 0.5% fetal bovine serum (FBS) and were subsequently induced with 20% FBS (see Fig. 4). At various times after induction, cells were harvested for cytoplasmic RNAs.

Polysome gradients and Northern blot analyses. Cell lysates for polysome analyses were made from pools of COS-1 cells containing stably integrated copies of the CS and α2-globin genes. Subconfluent plates of transformed cells were washed several times at 4°C with phosphate-buffered saline, 3×10^7 cells were lysed in 1 ml of buffer (8), and nuclei were pelleted at 14,000 × *g* for 10 min at 4°C. The supernatant ($2\,A_{260}$ units) was layered over 5 ml of linear 15% to 40% sucrose gradient, and polysomes were separated, as described previously (32), with a modified sucrose gradient buffer (10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.5], 10 mM KCl, 3 mM $MgCl_2$, 1 mM dithiothreitol, 0.2 mg of heparin per ml). Gradients were sacrificed by displacing them upwards through a UA5 UV monitor (ISCO Inc., Lincoln, Nebr.), and fractionation was based on the profile at an optical density at 260 nm. Individual fractions correspond to free mRNA; 40S, 60S, and 80S ribosomal subunits; disomes; and trisomes, etc. RNA (2 μg) isolated from each fraction was electrophoresed in a 1.2% agarose–6% formaldehyde–0.2 M morpholinepropanesulfonic acid (MOPS) gel. The RNAs were transferred to Gene Screen Plus membranes, UV cross-linked, prehybridized, and hybridized as described by the manufacturer (New England Nuclear). Probes for Northern (RNA) blots were generated by random primer labeling a 295-bp *Bst*EII-*Pst*I α2-globin gene fragment, a PCR-generated fragment which spans codons 130 to 235 of the rat c-*fos* cDNA (gift of L. S. Callans and W. Lee, University of Pennsylvania, Philadelphia), and a 400-bp *Hin*dIII-*Eco*RI fragment of the ribosomal protein rpL32 (39). RNA levels were determined by quantitating the signals from the Northern blots with a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.). The globin and c-*fos* signals were normalized for loading, as determined by the rpL32 ribosomal protein signal on the same Northern blot.

RT-PCR cloning assay. Primer extension reaction mixtures contained 5 to 10 μg of cytoplasmic RNA; 100 pmol of the N18(KpnI) primer (5′-GGGGTACCGCCCACTCAGACTTT A-3′), which is complementary to the last 18 bases of the

α-globin mRNA and has a *Kpn*I restriction site; 1 mM (each) deoxynucleoside triphosphates; 1× RT-PCR buffer (50 mM KCl, 20 mM Tris-HCl [pH 8.4], 1.5 mM $MgCl_2$); and 10 U of RT (Life Sciences, Gaithersburg, Fla.) in a 20-μl volume. These samples were amplified in 100 μl of the reaction mixture containing 10% dimethyl sulfoxide, 8 mM mercaptoethanol, 100 pmol of the ^{32}P-5′-end-labeled Tx+1(*Eco*RI) primer (5′-GGGAATTCACTCTTCTGGTCCCCA-3′, which is complementary to the first 16 bases from the transcription start site (Tx+1) of the α-globin cDNA and has an *Eco*RI site at the 5′ end), and 2.5 U of *Taq* polymerase (Perkin-Elmer Cetus, Norwalk, Conn.). Samples were subjected to one cycle of 5 min at 95°C, 1.5 min at 54°C, and 3 min at 72°C); 25 cycles of 1 min at 95°C, 0.5 min at 54°C, and 1.5 min at 72°C); 10-min extension at 72°C; and a final soaking at 4°C. To determine the ratios of mutant and α2-globin cDNAs in the total amplified material, the samples were digested with *Hin*dIII and *Kpn*I, and the 298-bp cDNAs (containing the normal translation termination codon or mutation) were gel purified and cloned into M13mp19. For the analysis of the ACG mutations, the 259-bp *Eco*RI-*Hin*dIII-digested fragments encompassing the normal translation start site (AUG) or the ACG mutation were gel purified and cloned into M13mp18. These digestion and gel purification steps ensure that the murine α-globin cDNA, which does not contain a *Hin*dIII site, as well as the genomic DNA, which is larger because of the presence of intron sequences, are excluded in the analyses. JM103 cells were transformed with the ligation mix, and replica lifts (500 plaques) were screened as described previously (59) with the ^{32}P-5′-end-labeled α2 oligonucleotide (5′-TCCAGCTTAAC GGTATT-3′) and a mutagenesis oligonucleotide. Replica lifts were then superimposed, and the plaques positive for each oligonucleotide were tallied. Because the mutant and normal α-globin mRNAs were reverse transcribed and amplified in the same tube with the same pair of primers, the relative levels of mRNA in the transfected cells were accurately reflected by the number of positive plaques. To verify that the screening was sufficiently stringent to detect a single-base change, four plaques (two positive and two negative for each screening oligonucleotide) were sequenced.

The ratios of mutant to α2-globin mRNAs obtained as raw data from the RT-PCR cloning assay were normalized for minor variations in concentrations of each plasmid DNA in the electroporation mix. Plasmid DNA concentrations in the electroporation mixes were determined by the PCR cloning assay or by cloning the 3′ halves of the plasmids into M13mp19 and screening with the oligonucleotides as described above. Similar ratios of mutant to normal plasmid DNAs were obtained with both techniques.

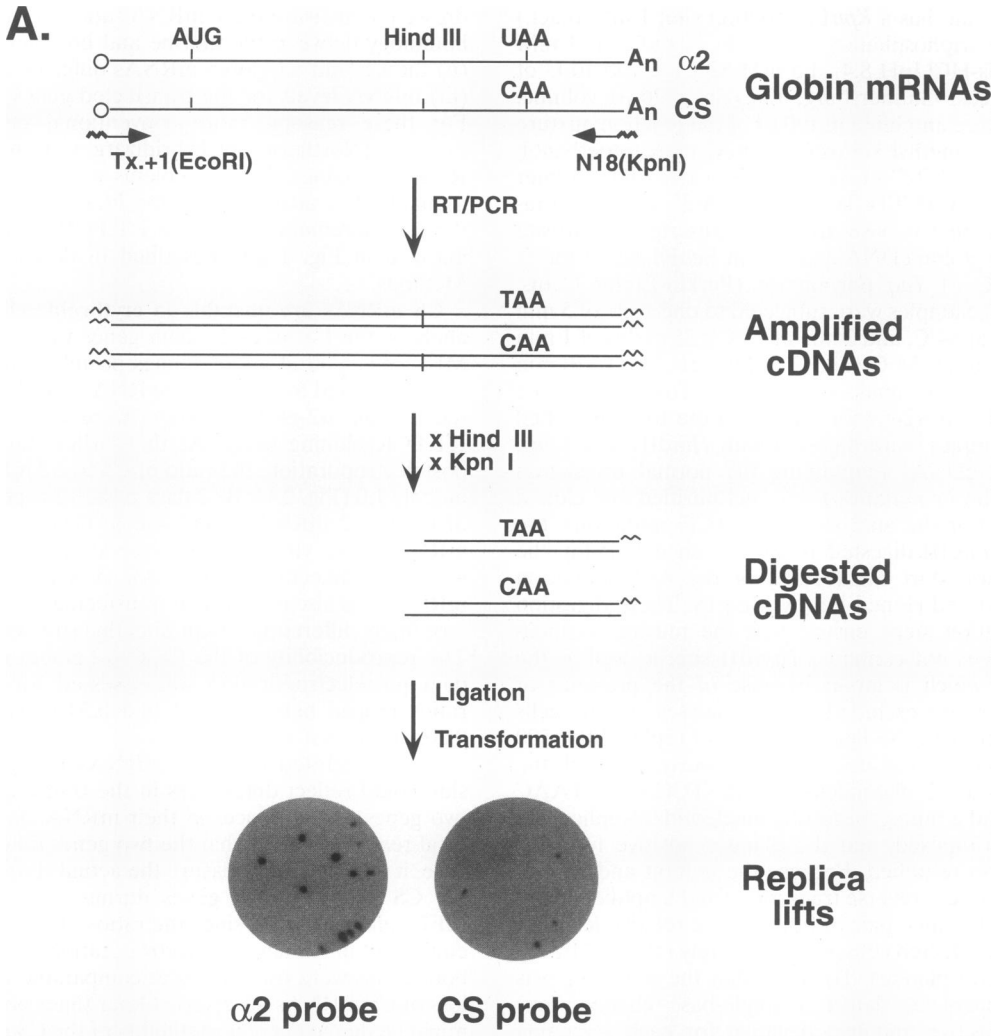
RESULTS

In order to study the mechanism responsible for the selective loss of CS mRNAs during RBC differentiation, we established an experimental system in which the expression of the mutant CS gene could be directly compared with that of the normal α2-globin control. Translation has been shown to have a major impact on the stability of some mRNAs. In addition, the CS mutation is in the translation termination codon (UAA→ CAA) and affects the pattern of translation. Therefore, the most important consideration in these studies was to compare expression of the CS and α2-globin genes within a uniform translational environment. To accomplish this, equal concentrations of the CS and α2-globin genes were coelectroporated into MEL cells, and the ratios of mutant to normal mRNAs were quantitated. Several technical problems had to be addressed to measure these mRNA ratios: (i) there is substantial homology between the murine and human α-globin mRNAs, (ii) the CS and α2-globin mRNAs differ by a single base, and (iii) mRNA levels for the transfected genes may be very low. For these reasons, more conventional methods were not practical (Northern blot hybridization, primer extension, and RNase mapping). These problems were overcome by utilizing a quantitative adaptation of the PCR (62), modified to meet these requirements (59). This RT-PCR cloning assay is summarized in Fig. 1 and described in detail in Materials and Methods.

CS mRNAs are unstable in erythroid cells. For the initial analysis, the CS and α2-globin genes were cotransfected into MEL cells, and at various times postelectroporation the cells were harvested for cytoplasmic RNAs and the relative levels of the CS and α2-globin mRNAs were examined by using the RT-PCR cloning assay. At the earliest time point, 0.3 day postelectroporation, the ratio of CS to α2 mRNA was approximately 1.0 (Fig. 2A). By 2 days postelectroporation, the ratios of CS to α2 mRNAs decreased to 0.34:1, and on day 6, CS mRNAs were virtually zero even though α2-globin mRNAs were still detectable (Fig. 2A). A similar decrease in CS mRNAs was also observed in transfected MEL cells induced to terminally differentiate with dimethyl sulfoxide (Fig. 2A) (16). The reproducibility of the CS-to-α2-globin mRNA ratio at 2 days postelectroporation was assessed for 12 samples; the ratios ranged between 0.1:1 and 0.34:1, with an average of 0.20:1 (data not shown).

The accelerated loss of CS mRNAs during transient expression could reflect differences in the transcription rates of the two genes or differences in their mRNA stabilities. For technical reasons, namely that the two genes differ at only a single base, it is difficult to measure the actual transcription rates for the CS and α2-globin genes during transient expression in MEL cells. However, since the ratios of the two mRNAs were equivalent at 0.3 day postelectroporation, it seemed likely that both genes were transcribed at comparable rates and that the loss of CS mRNAs observed at later times postelectroporation might be due to decreased stability of the CS mRNA compared with that of the wild-type α2-globin control. This was tested by examining the ratios of CS to α2-globin mRNAs in MEL cells transcriptionally inhibited with actinomycin D. In two separate experiments, 3-h incubations with actinomycin D caused a 2.5-fold decrease in the ratios of CS to α2 mRNAs, from 0.94:1 to 0.38:1 (Fig. 2B). These results demonstrate that CS mRNAs are less stable than α2-globin mRNAs in MEL cells. This drop in the ratio of CS to α2 mRNA in transfected MEL cells over time parallels the observation of an accelerated loss of CS mRNA in vivo relative to α2-globin mRNA as cells differentiate from transcriptionally active precursors to enucleated reticulocytes (24, 33), suggesting that the mechanisms responsible for the selective loss of CS mRNAs may be similar in the two systems.

CS mRNAs are stable in nonerythroid cells. To determine if the destabilization of CS mRNAs observed in MEL cells reflects an erythroid cell-specific mechanism, the levels of CS and α2-globin mRNAs were examined during transient expression in nonerythroid cells. Comparable levels of CS and α2-globin mRNAs were observed for 3 to 6 days following transfection in murine fibroblast (C127) cell lines (Fig. 3) and in COS-1 cells (data not shown). While the equal levels of CS and α2-globin mRNAs following transfection in nonerythroid cells suggest that the rates of transcription and mRNA degradation are similar, this experiment did not address whether the mRNAs are equally stable or equally unstable. Therefore, these genes were placed under the transcriptional control of



B.

INPUT CS: α2 PLASMID DNA RATIO

PCR CYCLES	2.5 : 1	1 : 1	1 : 2.5
15	2.5 : 1	1.1 : 1	1 : 2.3
30	2.1 : 1	1.3 : 1	1 : 2.5
45	2.2 : 1	1.2 : 1	1 : 2.4

FIG. 1. RT-PCR cloning assay. (A) Overview of the RT-PCR cloning assay. RNAs isolated from cells coelectroporated with the CS and α2-globin genes were reverse transcribed with primer N18(KpnI). The cDNAs were amplified and digested, and the gel-purified fragment was cloned into M13mp19 as shown. The *Hin*dIII digestion and gel purification steps were added to ensure that only human α-globin mRNAs were quantitated; the murine α-globin mRNA does not have a *Hin*dIII site and the amplified, digested α-globin gene would be larger because of the presence of intron II. This ligation mix was used to transform JM103 cells, and replica lifts (500 plaques for each datum point) were hybridized with ^{32}P-labeled oligonucleotides specific for the normal translation termination codon and the CS point mutation. Autoradiographs of the two replica lifts were superimposed, and the plaques positive for either the CS mutation or the α2 stop site were counted to obtain a ratio for the relative levels of the two mRNAs. (B) Quantitation control. The CS and α2 plasmid DNAs were mixed in 2.5:1, 1:1, and 1:2.5 ratios as shown. Each mix (50 ng) was amplified; aliquots were removed at 15, 30, and 45 cycles as noted, and the relative levels of CS and α2 at each cycle were quantitated by using the cloning assay. The experimentally derived CS-to-α2 ratios (shown in the box) accurately reflect the input plasmid DNA ratios for 45 amplification cycles. All RNA analyses were performed after 25 to 35 PCR cycles.

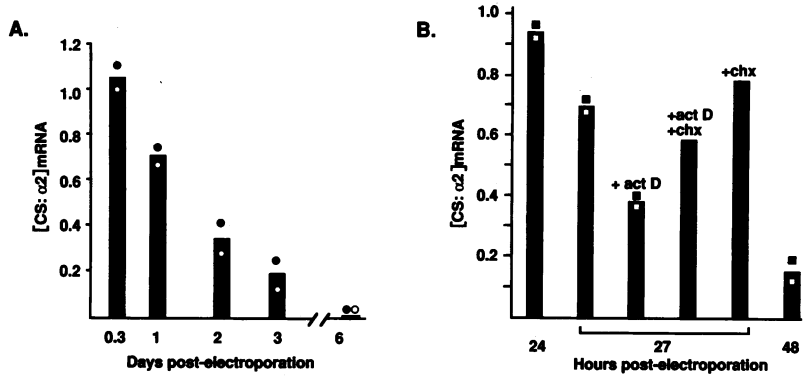


FIG. 2. CS mRNAs are unstable in erythroid cells. MEL cells were cotransfected with equal quantities of the CS and α2-globin genes. (A) At the indicated times postelectroporation, the CS-to-α2 mRNA ratios were determined by using the RT-PCR cloning assay as described in the legend to Fig. 1. For each time point tested, α2 mRNAs were defined as 1.0, and CS mRNAs were expressed relative to this value. Each histogram bar represents the average of two experiments, one in the presence (●) and one in the absence (○) of dimethyl sulfoxide, which induces MEL cells to terminally differentiate. (B) At 24 h postelectroporation, the cells were treated with actinomycin D (act D) and/or cycloheximide (chx) for 3 h. The ratios of CS to α2-globin mRNAs were determined by using the RT-PCR cloning assay (Fig. 1). When histogram bars represent the average of two experiments, data from the first experiment are noted by an open box. Control cells which received no drug treatment are represented by the unlabeled histogram bars.

the serum-inducible c-*fos* promoter and were used to establish stable cell lines which express either the CS or the α2-globin mRNA. These cell lines were made quiescent by a prolonged exposure to low levels of serum, and the *fos* promoter was then transiently activated with the addition of 20% FBS (18). Since heterologous genes under the control of the *fos* promoter are subject to a similar transcriptional burst (56), the stabilities of the CS and α2-globin mRNAs encoded by the chimeric genes can be determined by harvesting cells at various times after serum induction and quantitating the mRNA levels by Northern blot hybridization.

Figure 4 shows the results from this type of analysis. Endogenous c-*fos* mRNA levels were examined to monitor the experiment. The rapid accumulation and loss of the *fos* signal observed by Northern blot analysis are consistent with a transient induction of the promoter followed by decay of the unstable c-*fos* mRNA. As expected the CS and α2-globin mRNAs encoded by the chimeric genes also begin to accumulate around 0.5 to 1 h postinduction (Fig. 4A and B). However, in contrast to the unstable c-*fos* mRNAs, levels of the CS and α2-globin mRNAs do not decline rapidly after induction and are readily detectable up to 96 h later, indicating that the mRNAs are stable. Consistent with this, CS and α2-globin mRNAs were observed prior to transcriptional induction by high levels of serum (Fig. 4A and B, lanes labeled 0) and represent mRNAs which persisted through 3 days of culture in low levels of serum (in the absence of transcription). Similarly, baseline levels of stable *fos*/β-globin chimeric mRNAs have also been observed after prolonged incubation under serum starvation conditions (21). While these baseline levels of globin mRNAs make it difficult to accurately determine the half-lives of the CS and α2-globin mRNAs, the slow decline in the concentrations of the CS and α2-globin mRNAs within the first 8 h postinduction suggests that these mRNAs are relatively stable in this nonerythroid cell line (Fig. 4C and D). These findings indicate that erythroid and nonerythroid cells metabolize the mutant CS mRNAs differently. In MEL cells and human RBC, the CS mRNAs are targeted for accelerated degradation, while in the nonerythroid C127 and COS-1 cells, the CS and α2-globin mRNAs are metabolized at similar rates.

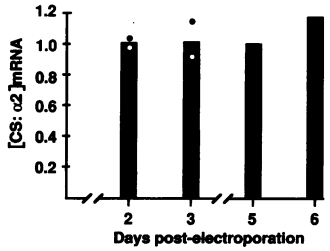


FIG. 3. CS and α2 mRNA levels were comparable during transient expression in nonerythroid cells. C127 (murine fibroblast) cells were cotransfected with equal quantities of the CS and α2-globin genes. At the indicated times postelectroporation, the CS-to-α2 mRNA ratios were determined by using the RT-PCR cloning assay (as detailed in Fig. 1). For each time point tested, α2 mRNAs were defined as 1.0, and CS mRNAs were expressed relative to this value. When histogram bars represent two experiments, data from the 3- and 6-day time courses are indicated by open and closed circles, respectively.

Translation and mRNA stability. Translation is an integral part of mechanisms mediating the degradation of many mRNAs (7). Partial stabilization of the CS mRNA in MEL cells treated with cycloheximide (Fig. 2B) suggested that translation was also required for its accelerated turnover. This could reflect involvement of a labile protein in the degradation pathway and/or a need for translation of the CS message. To distinguish between these two possibilities, translation of the CS mRNA was specifically inhibited by introducing a second site mutation at the initiation codon (AUG→ACG) of the CS mRNA (ACG.CS). The next start site in a good context (28) is in the +1 frame at codon 24, and the new open reading frame would terminate 24 codons downstream. Additional AUGs at codons 32 and 76 are in poor contexts for initiation. This ACG.CS gene was coelectroporated with the α2-globin gene into MEL cells, and mRNA levels were determined 2 days later. Inhibiting translation of the CS mRNA by this second site mutation (ACG.CS) resulted in significant stabilization of the message (Fig. 5). In contrast, the ACG mutation had a minimal impact on the level of wild-type α2 mRNA at 2 days postelectroporation (Fig. 5, ACG.α2). These results couple

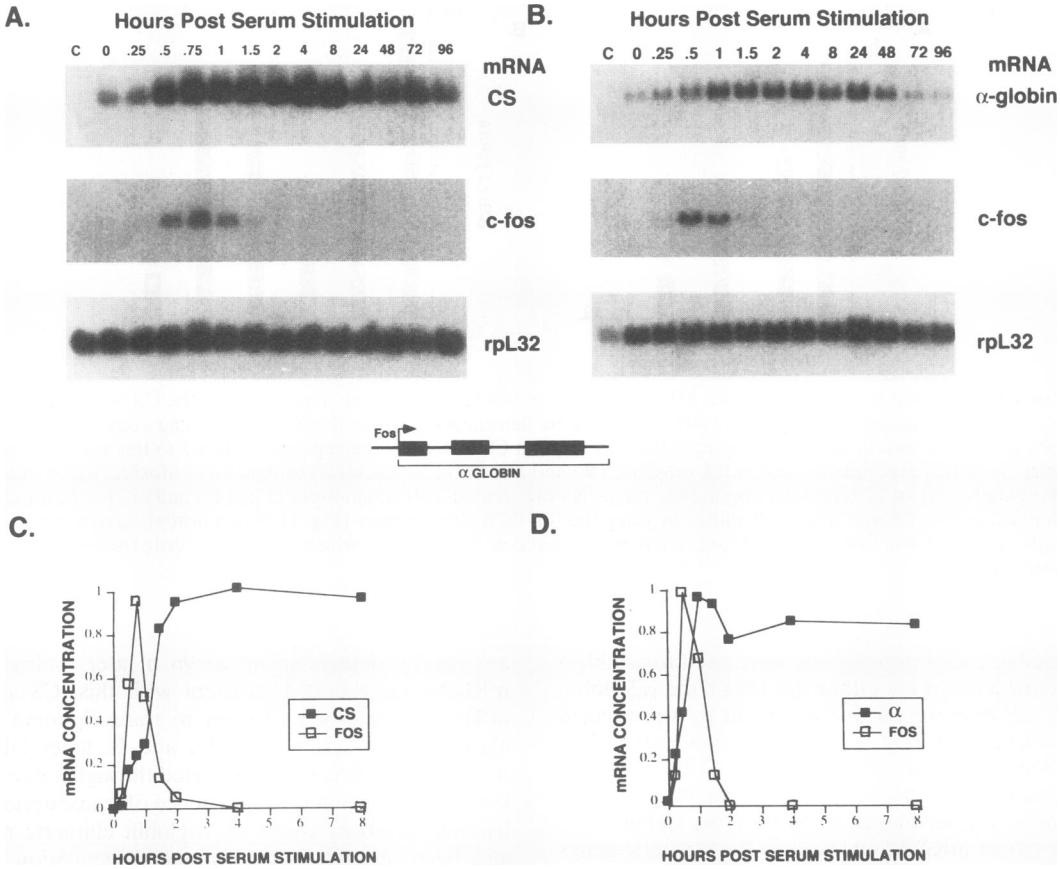


FIG. 4. CS and α2 mRNAs are stable in the nonerythroid C127 cells. Pools of C127 cells stably transformed with the chimeric *fos*/CS (A) or *fos*/α2 (B) gene were cultured for 3 days with 0.5% FBS and induced with 20% FBS. At the indicated times postinduction, the cells were harvested for RNA, and 5 μg of RNA was analyzed by Northern blot hybridization. The serum starvation/induction was performed twice on the same cell line, with identical findings, and the Northern blot data from one set of experiments are shown. A nontransfected C127 control RNA (lane C) is shown on the left. Each blot was hybridized sequentially with random primer-labeled probes to detect mRNAs for CS and α2-globin, ribosomal protein (rpL32), and c-*fos* as noted. Exposure times for the hybridizations were 16 h (α-globin), 1 h (rpL32), and 16 h (*fos*). RNA levels were determined by quantitating the signals from the Northern blots with a PhosphorImager (Molecular Dynamics). Samples were normalized for loading, and the highest value postinduction was defined as 1. To plot the concentrations for the CS (C) and α2-globin mRNAs (D), the amount of signal present at time zero was subtracted from that at each time point.

translation of the CS mRNA with its accelerated turnover in MEL cells.

Given the link between translation of the CS mRNA and its destabilization in MEL cells, it was important to determine whether the stability of CS and α2 mRNAs in nonerythroid cells resulted from an inability to translate globin messages. This question was addressed by assessing the polysomal distribution of α-globin mRNAs in stable COS-1 cell lines expressing the CS and α2-globin genes (Fig. 6). RNA was extracted from the indicated sucrose gradient fractions, and α-globin mRNA content was assayed by Northern blot analysis. The distribution of α-globin mRNAs from the electroporated COS-1 cells shows peaks of intensity in fractions containing two and three ribosomes per message and is identical to that observed for endogenous α-globin mRNAs in reticulocytes (54). An analysis of RNAs isolated from pooled polysomal fractions by the RT-PCR cloning assay showed comparable levels of CS and α2-globin mRNAs (data not shown). Efficient translation of α-globin mRNAs in COS-1 cells is in agreement with prior studies demonstrating translation of this message in a variety of other nonerythroid cells (22, 23). Thus, the longevities of CS and α2 mRNAs in nonerythroid cells do not

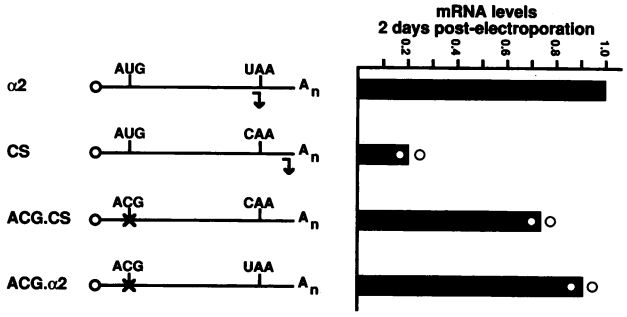


FIG. 5. Destabilization of CS mRNA is linked to its translation. MEL cells were coelectroporated with equal quantities of the mutant and normal α2-globin genes as noted, and relative mRNA levels were determined at 2 days postelectroporation by using the RT-PCR cloning assay (Fig. 1). For each experiment, α2-globin mRNA levels were defined as 1, and mutant mRNA levels were expressed relative to this value. Histogram bars represent the average of two experiments, marked by circles. Identities of the mRNAs are shown on the left; the cap site (o), translation start site (AUG) or mutation (ACG), stop codon (UAA) or mutation (CAA), and polyadenylation site (A_n) are labeled. The location of translation termination is shown by an arrow below the mRNA.

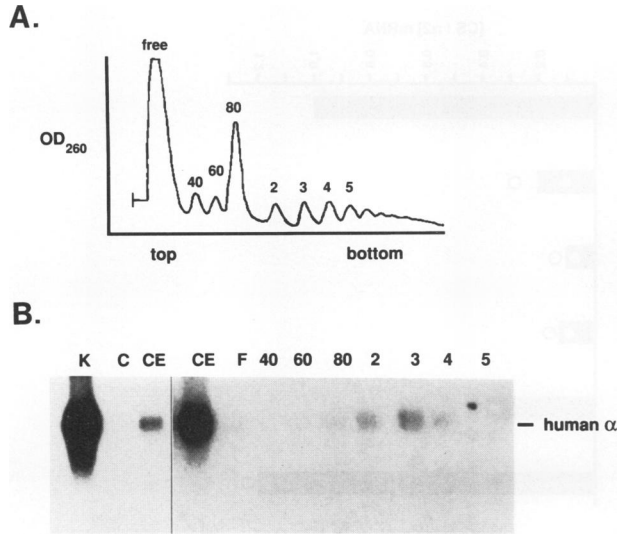


FIG. 6. α-Globin mRNAs are translated in nonerythroid COS-1 cells. (A) Cytosolic extracts of COS-1 cell lines containing stably integrated copies of the CS and α2-globin genes were subjected to sucrose gradient centrifugation to separate polysome fractions. (B) RNA (2 μg) from each peak was analyzed by Northern blot hybridization with an α-globin cDNA probe. The polysome profile at an optical density of 260 nm (OD_{260}) is shown in panel A. Fractions: free, free mRNA; 40 and 60, the 40S and 60S ribosomal subunits, respectively; and 80, 2, and 3, monosomes, disomes, and trisomes, respectively; 4 and 5, polysomal fractions. In panel B, control samples include 3 μg of RNA from K562 (K) human erythroleukemia cells, nonelectroporated COS-1 cells (C), and stably transformed COS-1 cell lines containing the CS and α2-globin genes (CE). A 12-h exposure of the control lanes (left three lanes) and a longer, 7-day exposure of the CE control and polysomal fractions (nine lanes on the right) are shown. The presence of CS and α2-globin mRNAs in the pooled polysomal fractions was verified by the RT-PCR cloning assay. The position of the human α2-globin gene (human α) is indicated on the right.

result from sequestering these mRNAs from the translational machinery.

α-Globin mRNA destabilization is triggered by entry of the translating ribosome into the 3′ NTR. Destabilization of the CS mRNA in MEL cells could be mediated either by the UAA→CAA base substitution per se or by the loss of translation termination at that site. This issue was addressed by analyzing the expression of three additional naturally occurring α2-globin mutants. Two of these mutations, KD (UAA→UCA) and Icaria (UAA→AAA), like CS, are single-base changes in the stop codon which allow the ribosome to translate an additional 31 codons into the 3′ NTR (11, 14). Each mutant gene was coelectroporated into MEL cells with an equal amount of the α2-globin gene, and the relative mRNA levels were determined 2 days later. The concentrations of both KD and Icaria mRNAs were 5- to 8-fold-lower than the α2-globin mRNA (Fig. 7A), suggesting that these RNAs were also targeted for accelerated degradation. The third mutation, Wayne, is a 1-base deletion (TCC→TC-) in exon 3 which frameshifts the ribosome past the intact α2 stop site before terminating five codons into the 3′ NTR (19). At 2 days postelectroporation, Wayne mRNA levels were reduced by 2.5-fold relative to the normal α2-globin mRNA levels (Fig. 7A).

Since all of these mRNAs were less stable than the wild-type message and the point mutations which allowed translational readthrough were different in each case, the data suggest that mRNA destabilization was not dependent on the specific base substitution but instead reflects a more general phenomenon associated with antitermination of α2-globin mRNAs. Accordingly, we would expect to stabilize the CS mRNA by inhibiting the translating ribosome from entering the 3′ NTR. A translation stop signal was introduced two codons 5′ to the CS mutation (CS/−2). In this mRNA, translation terminates just upstream of the CS mutation and the elongating ribosome would not enter the 3′ NTR. At 2 days postelectroporation, the CS/−2 mRNA was as stable as the normal α2-globin message in MEL cells (Fig. 7A).

The differential stabilities of the mutant Wayne mRNAs compared with the CS, KD, and Icaria mRNAs suggested that the extent of translational readthrough might correlate with the degree of mRNA destabilization. A series of genes were constructed to test this hypothesis. These genes contain both the CS mutation and an in-frame stop codon positioned between the CS mutation and the CS stop site at codon +31 (+ indicates the number of additional codons translated within the 3′ NTR). These mutations allow translation to continue 1, 4, or 14 codons into the 3′ NTR and are referred to as CS1, CS4, and CS14, respectively. As a control for effects of these 3′ NTR base substitutions on message stability, each mutation was also introduced into the normal α2-globin gene. This second set of mutations (designated U1, U4, and U14) encode mRNAs which terminate translation at the normal site.

Each of the derivative CS genes was coelectroporated with an equal quantity of the normal α2-globin gene into MEL cells, and the mutant-to-α2 mRNA ratios were determined 2 days postelectroporation (Fig. 7B). Translation one codon past the normal stop site (CS1) had a minimal effect on mRNA levels. When considered with the normal level of α-globin mRNAs observed if translation terminates two codons 5′ to the stop signal (Fig. 7A, CS/−2), this result suggested that some flexibility in the site of translation termination was compatible with normal message stability. In contrast, when translation terminated 4 or 14 codons into the 3′ NTR (Fig. 7B, CS4 and CS14), mRNA levels were substantially reduced relative to the α2-globin control level. Since the base substitutions at +4 and +14 had no significant effect on mRNA levels when the ribosome terminated at the normal position (mRNAs U4 and U14; data not shown), accelerated turnover of the CS4 and CS14 mRNAs was a consequence of the ribosome translating into the 3′ NTR. These results suggest that translational readthrough affects a determinant required for stability of the message in erythroid cells. Furthermore, translation must extend between one and four codons into the 3′ NTR to affect the function of this determinant.

DISCUSSION

We have developed a transient transfection system that in combination with an RT-PCR-based assay can be used to measure the impact of mutations on the stability of the α2-globin mRNA. In order to identify elements and mechanisms responsible for normal message stability, a subset of α2-globin mutations which lead to translational readthrough into the 3′ NTR has been analyzed. Several of these mutations, CS, KD, and Icaria, result in a complete loss of expression from the affected allele in vivo (11, 12, 14, 25, 40), while the Wayne mutation results in a moderately reduced accumulation of the mutant protein relative to the normal α2-globin (19). For the CS gene, the loss of expression correlates with the premature disappearance of the CS mRNA during erythroid differentiation (24, 32). Where data have been available,

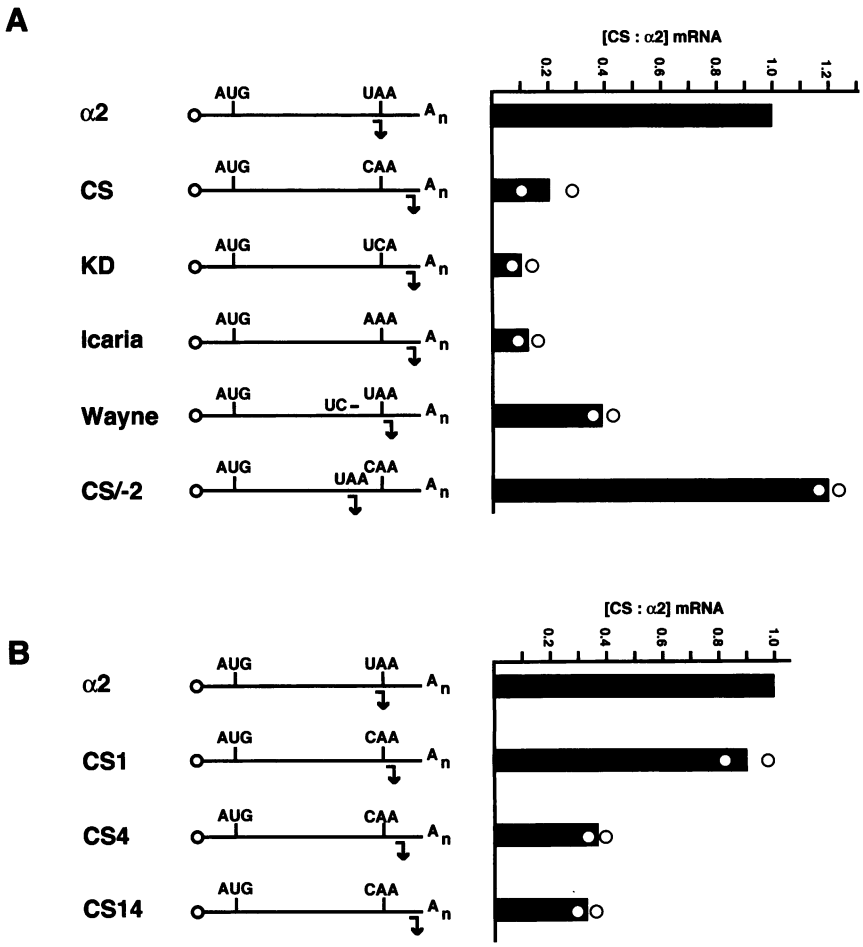


FIG. 7. α2-Globin mRNA is destabilized by entry of the ribosome into the 3′ NTR. MEL cells were coelectroporated with equal quantities of the mutant and normal α2-globin genes. At 2 days postelectroporation, the ratios of mutant to α2 mRNAs were determined by using the RT-PCR cloning assay (Fig. 1). The figure is labeled as described in the legend to Fig. 5. (A) Naturally occurring and site-directed mutant α2-globin genes; (B) translational readthrough mutants.

previously reported in vivo analyses of mRNA or protein levels have been consistent with the relative ordering of their mRNA stabilities during transient expression in MEL cells (this paper). This suggests that the degradation pathways for this group of mutant mRNAs are similar in human RBC and MEL cells and that this transient expression system is appropriate for further studies.

Initially, we analyzed the defect in expression of CS, a translation termination mutant (UAA→CAA). Experiments with MEL cells indicated that destabilization of the CS mRNA was mediated by entry of the translating ribosome into the 3′ NTR. Mutations which allowed the ribosome to readthrough as little as four codons into the 3′ NTR resulted in mRNA destabilization by 2 days postelectroporation (Fig. 7). Conversely, decreasing or blocking elongation into the 3′ NTR by inhibiting translation (Fig. 2B, +chx), mutating the initiation codon AUG→ACG (Fig. 5, ACG.CS), or preventing readthrough into the 3′ NTR by the introduction of a translation stop site (Fig. 7A, CS/−2) all increased stability of the CS mRNA.

Two different models could account for these results. Either translational readthrough is a general mechanism mediating message turnover or translational readthrough specifically destabilizes the α2-globin mRNA. The first model would predict that any message could be destabilized by readthrough. This is unlikely to be the case though, since CS1 mRNAs are stable in erythroid cells (Fig. 7B) and CS mRNAs are stable in nonerythroid cells (Fig. 4). In addition, a frameshift mutation in the β-globin gene which results in translational readthrough leads to equal ratios of mutant to normal proteins in vivo and, by inference, equal ratios of the respective mRNAs in erythroid cells (15). The second model predicts that a particular structure or determinant is associated with the normal α2-globin mRNA which is required for message stability in erythroid cells. In this case, the abnormal entry of the translating ribosome into the 3′ NTR disrupts this determinant, thereby targeting the message for accelerated turnover in erythroid cells.

While the data strongly suggest that translation readthrough mediates α-globin mRNA destabilization, the nature of the determinant which is affected is undefined in the present study. It could be composed of the poly(A)-poly(A)-binding protein complex. A ribosome terminating within the polyadenylation signal, as occurs in the CS, KD, and Icaria mRNAs, might alter this complex or affect the structure at the junction of the 3′ NTR and the poly(A) tail, leaving the mRNA more exposed to RNases. For the CS4 and Wayne mRNAs where translation termination occurs four or five codons into the 3′ NTR [about

93 nucleotides from the poly(A) tail], these structures may be less affected, accounting for their intermediate stability. In support of this mechanism, the poly(A)-poly(A)-binding protein complex has been shown to be an important component in determining β-globin mRNA stability in vitro (9), and some work implicates the poly(A) tail in determining α-globin mRNA stability (22, 23). However, if translational readthrough mediates α-globin mRNA stability by interfering with the poly(A)-poly(A)-binding protein complex in MEL cells, then it would also be expected to occur in the nonerythroid cells and lead to accelerated turnover of the CS mRNAs. Our results indicate that CS and α2-globin mRNAs are both relatively stable in nonerythroid cell lines (Fig. 4). Thus, while we cannot rule out a potential role for deadenylation in the degradation process, it is unlikely that a selective loss of the poly(A) tail in CS mRNAs would account for the accelerated turnover in erythroid cells, unless additional factors or RNases are involved in determining tissue specificity.

An alternative possibility is that the determinant is composed of specific sequences or elements in the α2-globin mRNA which form structures or mRNA protein complexes involving the 3′ NTR. Implicit in this model is the assumption that a determinant associated with the 3′ NTR could remain intact during translation of the normal α-globin mRNA and would be disrupted by translational readthrough. Two lines of evidence suggest that this is the case. Previous work from this laboratory has shown that a cDNA hybridized to the 3′ NTR of the human α-globin mRNA remains bound to the message during translation in rabbit reticulocyte lysates (54). Furthermore, the addition of a suppressor tRNA to the system allowed translational readthrough and resulted in dissociation of the cDNA from the α-globin message. Consistent with this data, the 3′ termini of rabbit α-globin mRNAs, isolated from polysomal fractions, were resistant to single-strand RNases, suggesting that the region was involved in a base-paired structure which was maintained during active translation (29).

One of the most intriguing results from these studies is the finding that translational readthrough has no detrimental effect on the half-lives of CS mRNAs in nonerythroid cells, raising the possibility that erythroid cell-specific *trans*-acting factors and/or degradation pathways are involved in determining α-globin message stability. For instance, the formation of an mRNA protein complex or structure could protect the normal α-globin message from degradation by an erythroid-specific RNase. Translational readthrough might dislodge such a factor or expose a target site, leading to accelerated metabolism of the CS message. Several reports have indicated that erythroid cells have degradation pathways which show a preference for particular mRNAs or sequences (4, 30, 31, 37, 61). Tissue-specific RNases may be particularly relevant to the erythroid lineage, where substantial mRNA turnover accompanies RBC differentiation. Our studies have identified a subset of mutant α2-globin mRNAs which are targeted for accelerated degradation in erythroid cells by virtue of a translating ribosome entering the 3′ NTR, suggesting that normal α-globin mRNAs contain signals or structures which compose a determinant required for their stability in erythroid cells. Translational readthrough results in a loss of the functional determinant, thereby leading to accelerated mRNA metabolism. The presence of an intact determinant could be one way that differentiating erythroid cells distinguish globin from nonglobin mRNAs. Conceivably, subsets of mRNAs containing this determinant could be protected from RNases, contributing to their accumulation during RBC differentiation.

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